Effects of Pharmacological Inhibition of AMPARs in the NAc and BLA on the Reinforcing Properties of Alcohol in Male C57BL/6J Mice

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Abstract

Alcohol Use Disorder is a national public health problem affecting over 16 million Americans. Prior research shows that molecular subunits of a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), expressed in reward-related brain regions, regulate the reinforcing effects of alcohol. Specifically, alcohol intake increases the expression of the AMPAR subunit GluA2 in the nucleus accumbens (NAc) and also the expression of the AMPAR subunit GluA1 in the basolateral amygdala (BLA). This suggests to us that GluA2-containing AMPARs in the NAc and GluA1-containing AMPARs in the BLA help with the regulation of the positive reinforcing effects of alcohol. In this experiment, we determine if the pharmacological inhibition of GluA2-containing AMPARs in the NAc or GluA1-containing AMPARs in the BLA affects the reinforcing effects of alcohol. We also sought to explore the alcohol specificity of this finding by evaluating AMPAR subunit regulation of the reinforcing effects of a non-drug reward compared to alcohol by using a sucrose control. Male C57BL/6J mice were trained to self-administer ethanol or sucrose in operant conditioning chambers and implanted with injector guides in the NAc or BLA. Mice were infused with pep2M, a GluA2 inhibitor, into the NAc to inhibit GluA2-containing AMPARs or NASPM, a GluA1 inhibitor, into the BLA to inhibit GluA1-containing AMPARs. Ethanol-reinforced responses were significantly reduced by pep2M in the NAc while sucrose-reinforced responses were unaffected. For NASPM in the BLA, both sucrose- and ethanol-reinforced responses were significantly reduced. These results suggest that GluA2-containing AMPARs in the NAc specifically regulate alcohol reinforcement but GluA1-containing AMPARs in the BLA regulate general reward processes. Our results indicate that alcohol may have specific effects on GluA2-containing

AMPARs in the NAc that drives the reinforcing, or addictive, effects of the drug. Supported by: R37-AAD14983

Introduction

Alcohol Use Disorder (AUD), a chronic relapsing brain disease characterized by uncontrolled alcohol consumption, is considered to be a global health issue. Worldwide, there are 2.5 million deaths annually due to harmful alcohol use (Wackernah et al., 2014). There are very few medications approved for treatment of AUD, showing a need for further research in order to develop more effective treatments.

As a sedative hypnotic, alcohol increases activity of inhibitory neurotransmitter systems such as gamma aminobutyric acid (GABA) (EŞEL & DİNÇ 2017). Chronic inhibition of GABA through chronic alcohol consumption promotes the neuroadaptive process of increasing the activity of excitatory glutamate pathways in order to try to restore brain homeostasis. The persistent hyperactivity of the glutamate system is a contributor to alcohol dependence and continuation of alcohol seeking behavior (Koob 2003). Since increased glutamate system activity promotes alcohol seeking behavior, this means that these pathways must regulate the reinforcing effects of alcohol (Rassnick et al. 1992.) The research on the regulation of alcohol reinforcement by glutamate signaling has primarily focused on the ionotropic subtype N-methyl-D-aspartic acid (NMDA) receptors and metabotropic receptors (Cannady et al. 2012). However, there is another type of ionotropic receptor called ɑ-amino-3-hyfroxy-5-methyl-4-isoxazolepropionic receptors (AMPARs) whose role in alcohol reinforcement has not been extensively studied as much as the other types of glutamate receptors. So far, there is already a significant amount of research showing that AMPARs play a role in the regulation of the reinforcement of other drugs of abuse, such as cocaine and heroin (Choi et al. 2011) (Van de Oever et al. 2008).

AMPARs are heterotetrametric cation channels and have four different subunits (GluA1-4). Not all AMPARs are composed of all four subunits and subunit composition affects the activity of the AMPARs (Jiang et al. 2007). Research on different AMPAR heteromers has focused on the nucleus accumbens because it is the main reward center. AMPARs that contain the GluA2 subunit, referred to as GluA2-containing AMPARs, in the NAc have shown to regulate cocaine-seeking behavior (Famous et al. 2008). Additionally, research has shown that alcohol increases GluA2 subunit expression (Faccidomo et al. 2020). These two findings together suggest the possibility of GluA2-containing AMPARs in the NAc playing a role in the regulation of the reinforcing properties of alcohol.

As the main reward center, the NAc is the convergence site for numerous glutamatergic projections coming from other reward-related regions such as the basolateral amygdala (BLA). Alcohol intake has shown to increase AMPAR activity in the BLA, suggesting that AMPARs in this region could also play a role in alcohol reinforcement (Salling et al. 2016). Additionally, alcohol consumption increases the expression of pGluA1 S831, a subtype of the GluA1 subunit, in the BLA (Cannady et al. 2017). These two findings together suggest the possibility that GluA1-containing AMPARs in the BLA regulate the reinforcing effects of alcohol.

In our lab, we are interested in knowing if GluA2-containing AMPAR activity in the NAc and GluA1-containing AMPAR activity in the BLA are essential for the reinforcing effects of alcohol. A self-administration method with C57BL/6J mice will be used because they are a common strain used in preclinical animal research of alcohol drinking since they are one of the only strains of mice that preferentially and voluntarily consumes large amounts of alcohol (Tabakoff, B. and Hoffman, P., 2000). The goal of this study is to determine if the inhibition of GluA2-contaning AMPARs in the NAc or GluA1-containing AMPARs in the BLA will modulate the reinforcing properties of alcohol. We will take a pharmacological approach into the investigation of these AMPARs, using the compounds pep2M and NASPM to inhibit AMPAR activity. Pep2M has been used to inhibit the GluA2 subunit and so will be used to inhibit GluA2-containing AMPAR activity (Lüthi et al. 1999). NASPM has been used to inhibit the GluA1 subunit and so will be used to inhibit GluA1-containing AMPAR activity (Zhang et al. 2019). The study will be replicated using sucrose, a general reward, in order to determine if findings pertain solely to the regulation of alcohol reinforcement or any reward. Finding an AMPAR that can be targeted in a specific brain region and solely affects the reinforcing properties of alcohol could be a beneficial target for the development of future pharmacological treatments of AUD.

Materials and Methods

Subjects

Male C57BL/6J mice (n=30) arrived from Jackson Laboratory (Bay Harbor, ME) at the age of 10 weeks and were group-housed (4/cage) in polycarbonate cages (28 x 17 x 14 cm). All cages were lined with corn cob bedding and included a cylindrical PVC pipe (8 cm long x 5 cm wide) and cotton nestlet for environmental enrichment. Mice were given access to Purina rodent chow and water ad-libitum, unless noted. The vivarium was held in a 12hr: 12hr reverse light/dark cycle vivarium (lights on at 19:00) that held a constant temperature of 21 ± 1^{0}C and humidity of $40 \pm 2\%$. Mice were given 1 week to acclimate to the reverse light/dark cycle. All procedures done were in accordance with the NIH Guide to Care and Use of Laboratory Animals (2011) and in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Carolina at Chapel Hill.

Self-Administration Equipment

Self-administration sessions were conducted in operant conditioning chambers (Med Associates, St. Albans, VT) housed inside a sound-deadening cubicle with a 28-V fan to hide external noise and ventilate the chamber. The front and roof panel of the chamber was made of clear plexiglass while the back and side walls were made of metal. The floor of the chamber was composed of steel rods with big enough gaps between them that any defecation could fall through into the bedding placed on the disposable bottom panel. The opposite walls of the chamber both had highly sensitive stainless-steel retractable levers that were placed under cue lights. Responding for only one of the levers (the “active” lever) was reinforced. Responses from the “inactive” lever were recorded to assess the general activity of the mice and the specificity of pressing the active lever. A drinking trough, adjacent to the active lever, was connected to a syringe pump outside the chamber. Mice were trained to press the active lever a certain number of times, depending on the Fixed Ratio (FR) they were on before 0.014 mL of the reinforcement was administered into the drinking trough. After the delivery of the reinforcement, there was a brief time-out period (800 ms) when pressing of the active lever was non-contingent. The chambers and pumps were all connected to an interface and computer to record the behavioral output of each operant chamber session for each mouse (MED-PC for Windows v.4.1).

Self-Administration Training Procedure

One week after arrival, mice (N=30) were given access to either 9% Ethanol (v/v) + 2% Sucrose (w/v) (ethanol) or 2% Sucrose (w/v) only (sucrose) solution, along with water, in their home cages for 2 weeks. This procedure allows the mice to be familiarized with the reinforcing solution before operant chamber self-administration training (Faccidomo, S. et al. 2015). The ethanol solution is slightly-sweetened with sucrose because there is more consistent intake from the mice compared to unsweetened ethanol (Faccidomo, S. et al. 2009). Consistent intake was defined as empty drinking troughs at the end of self-administration sessions.

Two weeks after the start of home-cage self-administration, mice began overnight sessions in operant chambers. Mice were placed in operant chambers overnight for 16 hrs and mice performed operant responses (active lever pressing) that were reinforced by a delivery of 0.014 mL of 9% Ethanol (v/v) + 2% Sucrose (w/v) (ethanol) (n=14) or 2% Sucrose (w/v) only (sucrose) (n=16) solution into the drinking trough. For the overnight sessions, mice were water-restricted 23 hours prior to each session to promote initial lever pressing. During the first overnight session, the mice responded on a Fixed-Ratio 1 (FR1) schedule, meaning each active lever press resulted in the delivery of ethanol or sucrose solution. The second overnight session started at FR1 for the first 25 reinforcements and then moved to FR2, followed by FR4, both changing in increments of 25. The third and successive sessions were only 1 hr in duration and were carried out in the dark phase between the hours of 1200-1600, 6 days/week. After each session, the drinking troughs in chambers were checked to confirm all delivered solutions were consumed.

Stereotaxic Surgery

After 40 1 hr self-administration sessions in operant chambers, mice underwent stereotaxic surgery. Mice were anesthetized with isoflurane gas and placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA). Mice were divided into two groups for different implantations. The first group consisted of 17 mice (8 from ethanol group and 9 from sucrose group) that were implanted with a bilateral 6 mm 26-gauge cannula (Plastics One, Roanoke, VA) aimed for the nucleus accumbens (NAc) (AP +1.6 mm from bregma; ML ±0.75 mm from bregma; DV -3.8 mm from skull surface) with 1 mm injector length. The second group consisted of 13 mice (6 from ethanol group and 7 from sucrose group) that were implanted with a bilateral 6 mm 26-gauge cannula (Plastics One, Roanoke, VA) with a 2 mm injector length aimed for the basolateral amygdala (BLA) (AP -1.2 mm from bregma; ML ±3.3 mm from bregma; -2.6 mm from skull surface). All coordinates were calculated using a mouse brain atlas (Franklin and Paxinos 2001). After inserted, the cannulas were secured to the skulls with dental cement (Durelon; Butler Schein, Dublin, OH) and had obturators (inserts for cannulas) (Plastics One) placed into them so no debris could clog the cannulas. The mice were then placed on heating pads in cages and were monitored until they woke up. Mice were given liquid ibuprofen (15 mg/kg) immediately after surgery and once a day for 3 days post-surgery. Obturators were frequently removed from cannula for the first week after surgery to prevent blockage or scarring. Mice were given 1 week to recover before resuming ethanol or sucrose self-administration sessions. Mice began to be individually housed after surgery to prevent damage to the obturator and cannula.

Drugs

Ethanol 9% (v/v) with 2% sucrose

All ethanol solutions (v/v) were prepared by 95% ethanol (Pharmco Products Inc, Brookfield, CT) and diluted with water to 9% before sucrose was added.

Pep2M and NASPM

Both drugs were purchased from Tocris Bioscience. Pep2M is a peptide inhibitor that blocks the binding site of N-ethylmaleimide-sensitive fusion protein (NSF) to GluA2, thus can be used to inhibit GluA2-containing AMPARs. NASPM is a selective antagonist for the GluA1 subunit, thus can be used to inhibit the function of GluA1-containing AMPARs. Artificial cerebrospinal fluid (aCSF) was used as the vehicle for both compounds.

Microinjections & Self-administration

After ethanol and sucrose self-administrations reached stable levels of responding after surgery, sham injections began, which involved inserting the injector into the head mount and turning on the pump, without administration of the drug. Sham injections were done to habituate the mice microinjection process. After sham treatments, mice were immediately placed into operant chambers for 1 hr ethanol or sucrose self-administration sessions. Sham injections were repeated until stable operant responding after injections was observed in their self-administration sessions. After sham injections, mice were given microinjections of the vehicle, artificial cerebrospinal fluid (aCSF) in order for the mice to habituate to sensation of the microinjection. aCSF injections were repeated like shams until stable operant responding after injections was observed in their self-administration sessions.

Next, dose-effect curves for pep2M into the NAc of ethanol (n=8) and sucrose (n=9) mice and NASPM into the BLA of ethanol (n=6) and sucrose (n=7) mice (4 experimental groups). Pep2M (1.0 and 10.0 µg/0.5µL) or NASPM (5.6 and 10.0 µg/0.5µL) were injected in a counterbalanced design. Drug solutions were bilaterally-infused (0.5 µL/side) through two 1-µL

Hamilton syringes connected to a Harvard Apparatus pump (Holliston, MA), with a flow rate of 0.125 µL/min/side for a 4-min period. The injector remained in the cannula for an extra minute after injection in order to ensure that the entire dose diffused into the targeted brain region. During the microinjections, the mice were not restrained and were allowed to freely move around the cage. Immediately after, mice were placed into their individual operant chambers for a 1 hr sucrose self-administration session. Microinjections were performed at maximum of 2 times/week to minimize stress for the mice.

Microinjections & Locomotor Activity

Open-field activity was tested with a Plexiglas activity monitor chamber (27.9 cm^2; ENV-510, Med Associates) using two sets of 16 pulse-modulated infrared photobeams that recorded X-Y ambulatory movements every 100 ms to quantify the distance traveled (cm) throughout the session. Mice were habituated to the locomotor chambers in a 2 hr session prior to testing. After habituation, in a counterbalanced design, mice were injected with either aCSF and pep2M (10.0 µg/0.5µL) for NAc group or NASPM (5.6 µg/0.5µL for ethanol mice and 3.0 µg/0.5µL for sucrose mice) for BLA group before 1 hr locomotor sessions.

Perfusion & Histology

Promptly after the final microinjection and locomotor session, mice were deeply anesthetized with sodium pentobarbital (200 mg/kg) and were intracardially perfused with phosphate-buffered saline (PBS) and paraformaldehyde (PFA). Brains were post-fixed in PFA for 48 hrs, then sliced into 50 µm coronal sections with a vibratome (Leica VT1000S; Leica Microsystems, Buffalo Grove, IL). The slices were then mounted on gelatin-coated slides and stained with cresyl violet in order to visualize the injection site at x20 under a light microscope (Olympus CX_{41}; Olympus, Tokyo) for histological verification. Histological verification is still in progression and so stacked plates were only completed for sucrose mice. Currently, histological analysis has shown correct placement of all injectors for all sucrose mice (n=16) (Fig. 1).

Statistical Analysis

All analyses were performed using Prism v. 8.4 (GraphPad, La Jolla, CA). Responding on the active and inactive lever was analyzed using a 2-way Repeated Measures ANOVA (DOSE x TIME) for each dose of pep2M or NASPM. A Dunnett's multiple comparison test was used for post-hoc testing. Locomotor activity was analyzed using a 2-way Repeated Measures ANOVA (DOSE x TIME) for the lowest effective dose of pep2M or NASPM. A p-value under 0.05 represented significance in data and α was set to 0.5.

Results

Experiment 1: pep2M in NAc- Ethanol and Sucrose

Acquisition

Mice were split into two groups, with the first undergoing stereotaxic surgery to receive implantation of a cannula targeting the NAc (n=17). All mice in this group showed stable and comparable levels of ethanol (n=8) and sucrose (n=9) self-administration across the 40 sessions prior to stereotaxic surgery. At the end of each session, the drinking troughs were empty. The average number of reinforcements across the 40 drinking sessions did not differ significantly (15.7 ± 2.4 for ethanol and 16.1 ± 2.7 for sucrose group). Histological analysis under a microscope after brain slice staining showed that all sucrose-reinforced mice received injections into the region of the NAc (Fig. 1A). Histological analysis is still in progress for ethanol-reinforced mice.

Ethanol Self-Administration

Mice with cannula implantations directed at the NAc were microinjected with pep2M, a GluA2 inhibitor, in order to see if ethanol or sucrose operant responses would be affected by the inhibition of GluA2-containing AMPARs in this region of the brain. Intra-NAc microinjection of 10.0 μg/0.5μL pep2M decreased active lever pressing. A 2-way RM ANOVA test showed that there was a main effect of time (Fig. 2A; $F(11, 77) = 174.5$, $p<0.0001$) and that there was an interaction between dose and time (Fig. 2A; $F(22, 154) = 2.606$, $p=0.0003$). Dunnett's multiple comparisons for doses of pep2M vs. aCSF showed that the decrease in active lever pressing with the microinjection of 10.0 μg/0.5μL pep2M began to significantly decrease compared to aCSF at 20 min ($p=0.0178$) and increased significance throughout the rest of the session (Fig. 2A). A collapsed view of the active lever pressing represented in Figure 2B further showed that only the high dose of pep2M decreases active lever pressing. Inactive lever pressing was not affected significantly by pep2M at either dose (Fig. 6A).

Sucrose Self-Administration

Unlike with the ethanol group, intra-NAc microinjection of both doses of pep2M created no difference in active lever pressing during sucrose self-administration (Fig. 2C). A 2-way RM ANOVA test showed that there was no main effect of dose but showed there was a main effect of time (Fig. 2C; $F(11, 330) = 71.07$, $p<0.0001$) and subject (Fig. 2C; $F(30, 330) = 31.02$, $p<0.0001$). Additionally, there was an no interaction between dose and time (Fig. 2C). Dunnett's multiple comparisons for doses of pep2M vs. aCSF at any time during sucrose-self administration were not significant (Fig. 2C). A collapsed view of the active lever pressing represented in Figure 2D further showed that there was no significant change in active lever pressing for sucrose with microinjection of pep2M. Also, inactive lever pressing was not affected significantly by pep2M at either dose (Fig. 6C).

Locomotor

Open field activity was tested using locomotor chambers in order to see if microinjections affect the overall mobility of mice. The locomotor activity for both ethanol and sucrose mice were not affected by the microinjection of pep2M (Fig. 3). Two 2-way RM ANOVA tests showed that the main effect was time for both ethanol (Fig. 3A; $F(11, 288) = 75.44$, $p<0.0001$) and sucrose mice (Fig. 3C; $F(11, 192) = 48.51$, $p<0.0001$). The 2-way RM ANOVA test for the ethanol mice also revealed a slight effect from dose (Fig. 3A; $F(1, 288) = 6.138$, $p=0.0138$) but there was no interaction between time and dose (Fig. 3C; $F(11, 288) = 0.2234$, $p=0.9959$). Collapsed views of the distance travelled throughout the ethanol and sucrose mice sessions further showed that there was no significant change in locomotor activity with pep2M microinjection (Fig. 3B & D).

Experiment 2: NASPM in BLA

Acquisition

Mice were split into two groups, with the second undergoing stereotaxic surgery to receive implantation of a cannula targeting the BLA (n=13). All mice in this group showed stable and comparable levels of ethanol (n=6) and sucrose (n=7) self-administration across the 40 sessions prior to stereotaxic surgery. At the end of each session, the drinking troughs were empty. The average number of reinforcements across the 40 drinking sessions did not differ significantly (17.6 ± 4.9 for ethanol and 14.0 ± 2.9 for sucrose group). Histological analysis under a microscope after brain slice staining showed that all sucrose-reinforced mice received injections into the region of the BLA (Fig. 1B). Histological analysis is still in progress for ethanol-reinforced mice.

Ethanol Self-Administration

Mice with cannula implantations directed at the BLA were microinjected with NASPM, a GluA1 inhibitor, in order to see if ethanol or sucrose operant responses would be affected by the inhibition of GluA1-containing AMPARs in this region of the brain. Intra-BLA microinjection of NASPM decreased ethanol self-administration at both 5.6 and 10.0 µg/0.5µL doses (Fig. 4A and B).). A 2-way RM ANOVA test showed there was a main effect of dose (Fig. 4A; $F(2, 168) = 139.1$ $p<0.0001$). Additionally, the 2-way RM ANOVA test further showed along with a dose effect, there was a main effect of time (Fig. 4A; $F(11, 168) = 10.01$, $p<0.0001$) and that there was an interaction between dose and time (Fig. 4A; $F(22, 168) = 3.083$, $p<0.0001$). Dunnett's multiple comparisons for doses of NASPM vs. aCSF that the 10.0 µg/0.5µL of NASPM significantly decreased active lever pressing at an earlier time compared to the 5.6 µg/0.5µL (Fig. 4A). A collapsed view of the active lever pressing throughout the entire session showed a negative correlation with active lever pressing and dosage of NASPM (Fig. 4B). Also, inactive lever pressing was not affected significantly by NASPM at either dose (Fig. 6B).

Sucrose Self-Administration

Similar to the ethanol group, intra-BLA microinjection of 5.6 and 10.0 µg/0.5µL NASPM decreased sucrose self-administration (Fig. 4C and D). A 2-way RM ANOVA test showed that there was a main effect of dose (Fig. 4C; $F(3, 24) = 5.616$, $p=0.0046$). Additionally, the 2-way RM ANOVA test further showed along with a dose effect, there was a main effect of time (Fig. 4C; $F(11, 264) = 45.38$, $p<0.0001$) and that there was an interaction between dose and time (Fig. 4C; $F(33, 264) = 5.752$, $p<0.0001$). Dunnett's multiple comparisons for doses of NASPM vs.

aCSF that the 10.0 µg/0.5µL of NASPM significantly decreased active lever pressing at an earlier time compared to the 5.6 µg/0.5µL (Fig. 4A). A collapsed view of the active lever pressing throughout the entire session showed a negative correlation with active lever pressing and dosage of NASPM (Fig. 4D). Also, inactive lever pressing was not affected significantly by NASPM at either dose (Fig. 6D).

Locomotor

Open field activity was tested using locomotor chambers in order to see if microinjections affect the overall mobility of mice. The locomotor activity for both ethanol and sucrose mice were not affected by the microinjection of NASPM (Fig. 6). Paired t-tests showed that there was no significant difference in cumulative distance travelled between aCSF vs. 5.6 µg/0.5µL NASPM for ethanol mice (Fig. 6A; $p=0.9113$) as well as between aCSF vs. 3.0 µg/0.5µL NASPM for sucrose mice (Fig. 6C; $p=0.8609$). Two 2-way ANOVA test showed there was a main effect of time for both ethanol (Fig. 6B; $F(11, 72) = 4.593$, $p<0.0001$) and sucrose mice (Fig. 6D; $F(11, 108) = 26.51$, $p<0.0001$). The 2-way ANOVA test also revealed a slight effect from dose in ethanol (Fig. 6B; $F(1, 72) = 10.46$, $p=0.0018$) and sucrose mice (Fig. 6D; $F(1, 108) = 5.009$, $p=0.0273$) but there was no interaction between time and dose (Fig. 6B; $F(11, 72) = 0.1065$, $p=0.9999$) (Fig. 6D; $F(11, 108) = 0.4736$, $p=0.9159$).

Discussion

As a sedative hypnotic, alcohol modulates inhibitive neurotransmitter pathways, such as increasing the activity of GABA (EŞEL & DİNÇ 2017). As a neuroadaptive process to try to restore brain homeostasis, excitatory glutamate pathway activity is increased. This permanent change to hyperactivity of glutamate pathways after chronic alcohol consumption is a main contributor to why seizures is a symptom of withdrawal (EŞEL & DİNÇ 2017). There has been an extensive amount of research on how different glutamate receptors regulate reinforcement alcohol but there has been a lack on AMPARs compared to the other subtypes. The possibility of AMPARs playing a role in the reinforcing effects of alcohol looks promising since there is a significant amount of research showing they are used for the regulation of other drugs of abuse, such as cocaine and heroin (Choi et al. 2011) (Van de Oever et al. 2008).

In this study, it was shown that microinjection of 10.0 μg/0.5μL pep2M, a GluA2 inhibitor, into the NAc decreased active lever pressing for ethanol but not for sucrose (Fig. 2). The microinjection of pep2M did not affect locomotor activity of both groups of mice (Fig. 3). However, the microinjection of all doses of NASPM, a GluA1 inhibitor, into the BLA decreased active lever pressing for both ethanol and sucrose while locomotor activity was not affected (Fig. 4 & 5).

Previous research showed that enhanced AMPAR activity increases operant self-administration of ethanol, showing the AMPARs could play a role in the reinforcing effects of alcohol (Cannady, R. et al. 2013). Our results support this suggestion by showing that inhibition of the activity of specific AMPARs decreases operant self-administration of ethanol. Alcohol intake decreasing with the inhibition of these subunit-specific AMPARs supports literature showing that GluA2 protein expression in the NAc and GluA1 protein expression in the BLA play a role in alcohol reinforcement (Faccidomo et al. 2020) (Cannady et al. 2017).

The decrease in active lever pressing for ethanol with the microinjection of pep2M or NASPM could be interpreted as these pharmacological compounds make the mice move less, thus lowering their active lever pressing during operant sessions. However, locomotor activity was not affected by microinjection of pep2M or NASPM, supporting our conclusion that the decrease in active lever pressing comes from a change in the regulation of the reinforcing effects of alcohol instead of non-specifically also affecting locomotion (Fig. 3).

An unexpected result we saw was that active lever pressing for sucrose was only affected with the microinjection of NASPM in the BLA. Active lever pressing for sucrose was not affected by the microinjection of pep2M at any dose, suggesting that the GluA2-containing AMPARs in the NAc does not regulate the reinforcing effects for all rewards. This is different to the GluA1-containing AMPARs in the BLA, which appear to regulate reinforcing effects of all rewards since active lever pressing for ethanol and sucrose is significantly decreased with NASPM microinjections. The activity of the mice was not affected by the microinjections of these drugs, eliminating that the inhibition of these AMPARs affects physical movement of the mice as an alternative interpretation of these findings.

With the methodology used, we were not able to determine if cannulas were implanted into the correct brain regions after all experimentation. Brain slicing and staining has showed that sucrose-reinforced mice had correct injection sites but unfortunately, it is still unknown at this point of the research if the ethanol-reinforced mice had correct implantations (Fig. 1). Thus, a caveat of these findings is having to assume, until histological analysis can be completed, that injectors were implanted in the correct brain regions for ethanol-reinforced mice as well.

Another caveat of our methodology is the dose of NASPM injected into the BLA of the sucrose-reinforced mice during locomotor testing was the not the same as the dosage used for ethanol-reinforced mice. It is likely that this was due to an error in communication during experimentation set-up since ethanol and sucrose sessions happened on different days. In the future, our lab will replicate this study not only to gain a larger sample size, but to have locomotor testing with the same dosage of NASPM for the both groups.

Future endeavors after this study should include furthering research into GluA2-containing AMPARs in the NAc. Our findings suggest that these AMPARs in this particular brain region could solely play a role in the regulation of the reinforcing effects of alcohol and not just all rewards. If more research further proves this point, the GluA2-containing AMPARs could be a useful target for pharmacological treatment of AUD.

Conclusion

In this study, we showed that mice who were injected with pep2M into the NAc or NASPM into the BLA had a significant decrease in ethanol self-administration. From these findings, it can be concluded that both GluA2-containing AMPARs in the NAc and GluA1-containing AMPARs in BLA play a role in regulating the reinforcing effects of alcohol. These results also suggest that GluA2-containing AMPARs in the NAc solely regulate the reinforcing effects of alcohol while GluA1-containing AMPARs in the BLA regulates the reinforcing effects of any reward.

Figures

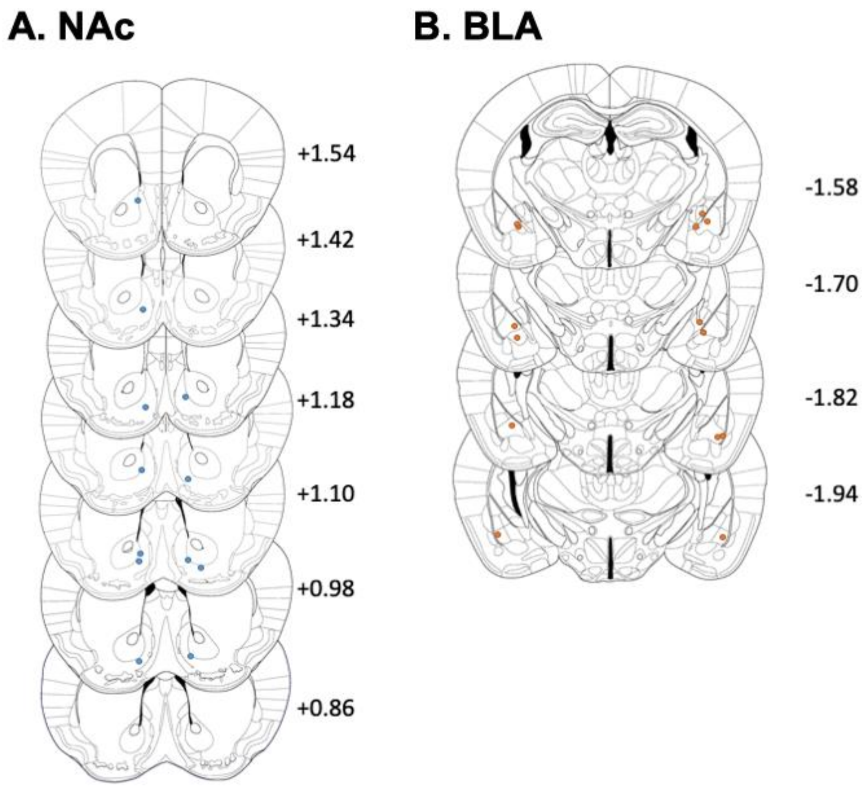

Figure 1. Stacked plate of injection sites for sucrose mice
A schematic representation of the mice showing A-P distance from Bregma. *Blue circles* indicate the approximate ventral tip of the injection site for each sucrose-reinforced mouse with injectors directed for the nucleus accumbens (NAc). *Orange circles* indicate the approximate ventral tip of the injection site for each sucrose-reinforced mouse with injectors directed for the basolateral amygdala (BLA).

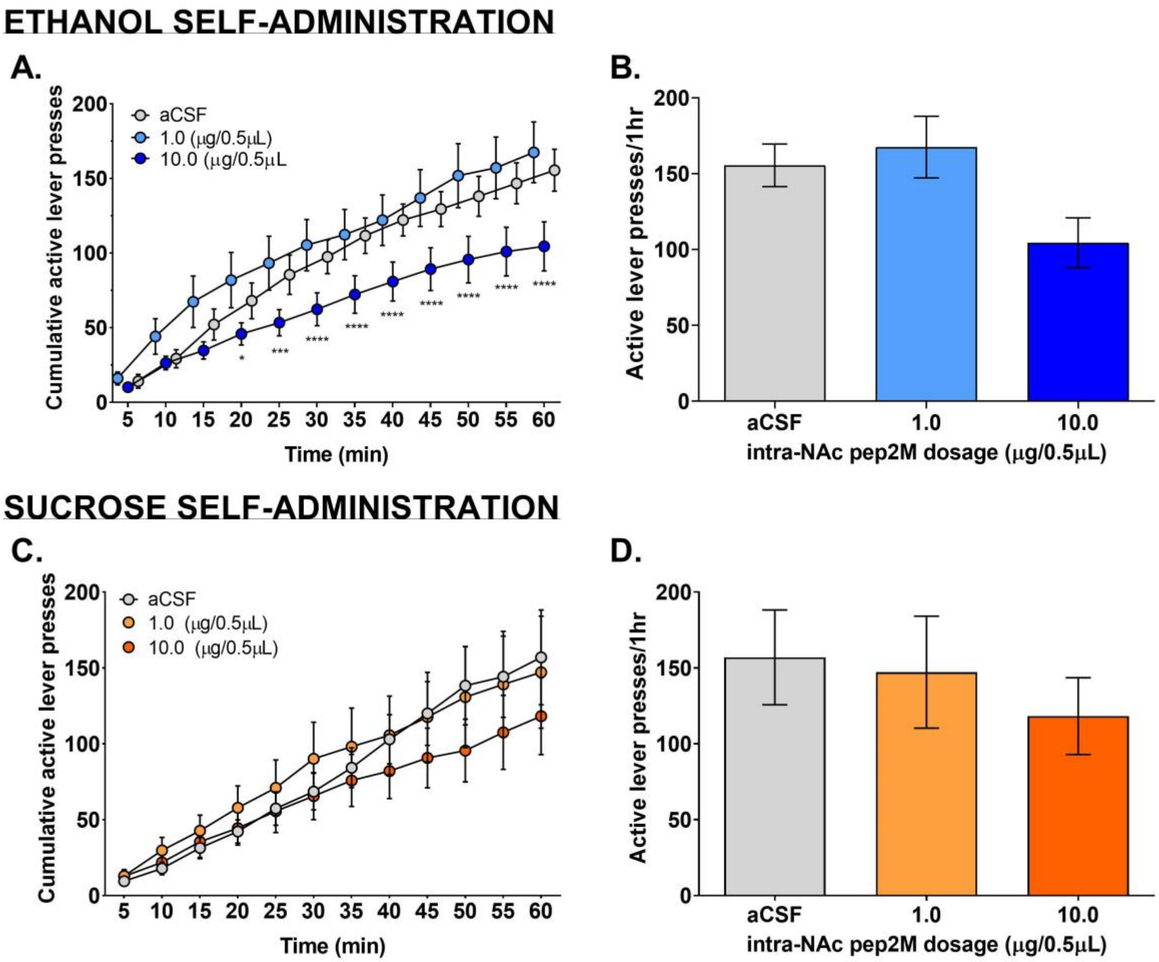

Figure 2. Operant responding after microinjections of pep2M into the NAc
(A) Active cumulative responses: *Grey circles* represent the cumulative number of ethanol active lever presses after vehicle (aCSF) microinjection. *Blue circles* represent the progressive effect of pep2M (1.0 and 10.0 μg/0.5μL) on the cumulative number of active lever presses for ethanol. **(B) Active lever presses:** The *grey vertical bar* represents the number of ethanol active lever presses after vehicle (aCSF) microinjection. *Blue vertical bars* represent the overall effect of pep2M (1.0 and 10.0 μg/0.5μL) on number of active lever presses for ethanol. **(C) Active cumulative responses:** *Grey circles* represent the cumulative number of sucrose active lever presses after vehicle (aCSF) microinjection. *Orange circles* represent the progressive effect of pep2M (1.0 and 10.0 μg/0.5μL) on the cumulative number of active lever presses for sucrose. **(D) Active lever presses:** The *grey vertical bar* represents the number of sucrose active lever presses after vehicle (aCSF) microinjection. *Orange vertical bars* represent the overall effect of pep2M (1.0 and 10.0 μg/0.5μL) on number of active lever presses for sucrose. In all panels, data are presented as mean (*vertical bars or circles*) ± SEM (*vertical lines*) and *asterisks* denote significance as compared to vehicle ($P<0.05$).

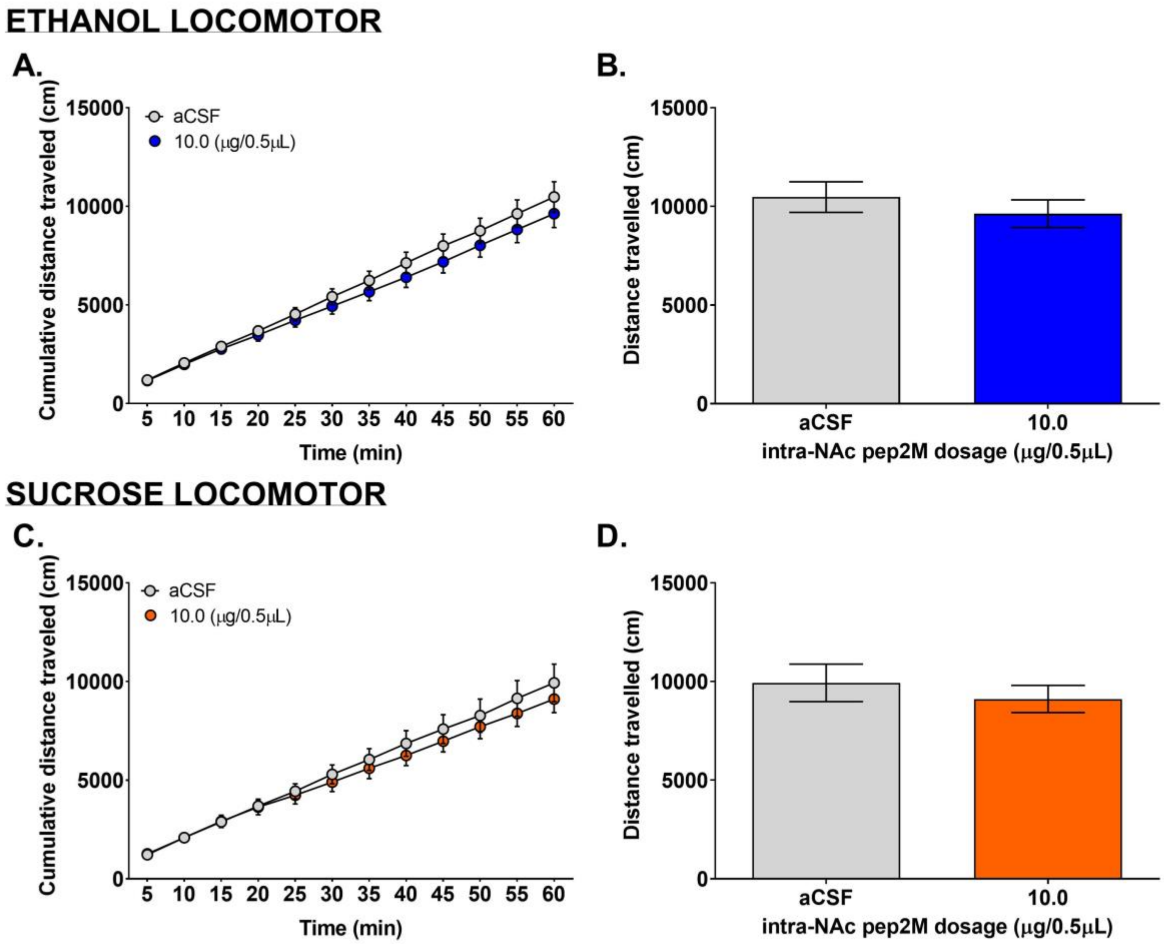

Figure 3. Locomotor activity after microinjection of pep2M into the NAc
(A) Cumulative distance travelled: *Grey circles* represent the cumulative distance travelled (cm) after vehicle (aCSF) microinjection. *Blue circles* represent the progressive effect of pep2M (10.0 μg/0.5μL) on the cumulative distance travelled (cm). **(B) Total distance travelled:** The *grey vertical bar* represents the total distance travelled (cm) after vehicle (aCSF) microinjection. *Blue vertical bar* represents the overall effect of pep2M (10.0 μg/0.5μL) on total distance travelled (cm). **(C) Cumulative distance travelled:** *Grey circles* represent the cumulative distance travelled (cm) after vehicle (aCSF) microinjection. *Orange circles* represent the progressive effect of pep2M (10.0 μg/0.5μL) on the cumulative distance travelled (cm). **(D) Total distance travelled:** The *grey vertical bar* represents the total distance travelled (cm) after vehicle (aCSF) microinjection. *Orange vertical bar* represent the overall effect of pep2M (10.0 μg/0.5μL) on number of active lever presses for sucrose. In all panels, data are presented as mean (*vertical bars or circles*) ± SEM (*vertical lines*) and *asterisks* denote significance as compared to vehicle ($P<0.05$).

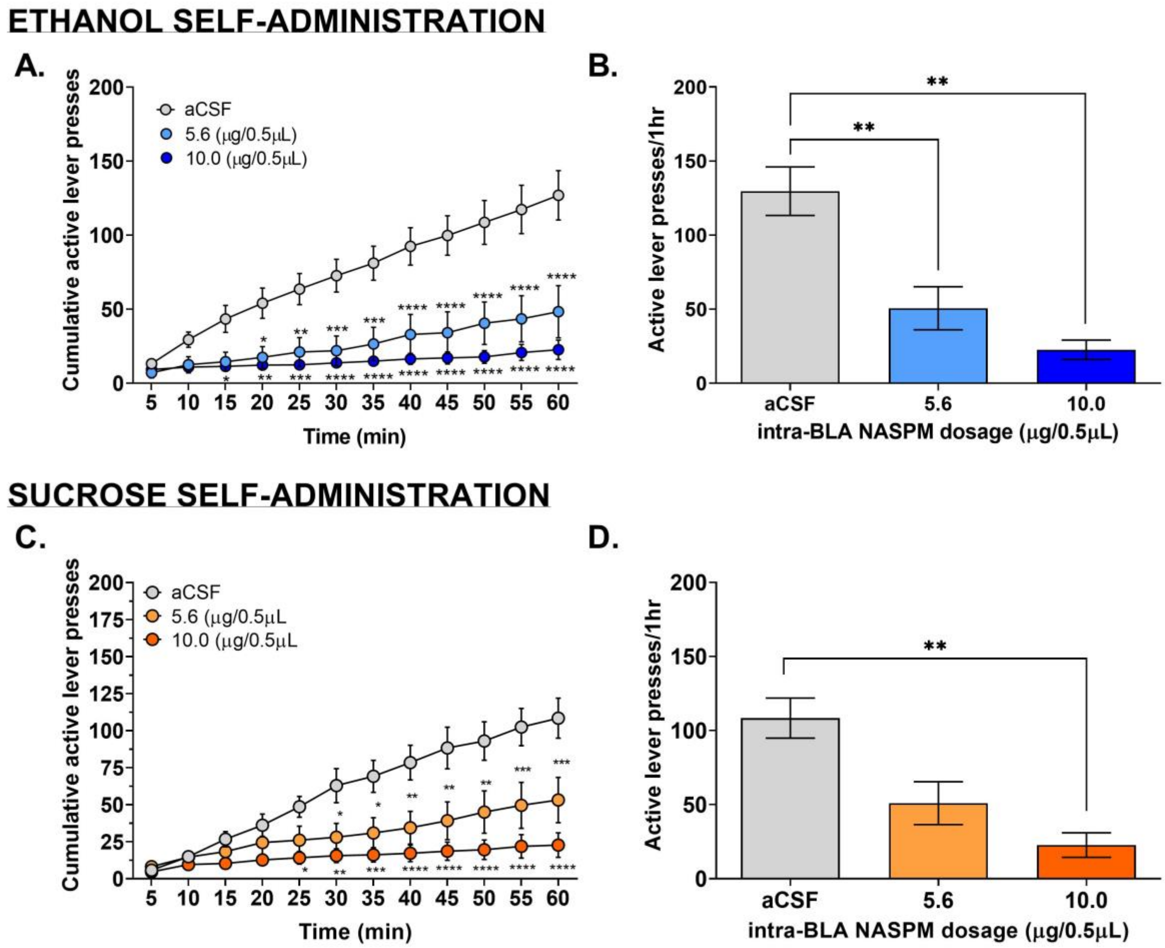

Figure 4. Operant responding after microinjection of NASPM into the BLA
(A) Active cumulative responses: *Grey circles* represent the cumulative number of ethanol active lever presses after vehicle (aCSF) microinjection. *Blue circles* represent the progressive effect of NASPM (5.6 and 10.0 µg/0.5µL) on the cumulative number of active lever presses for ethanol. **(B) Active lever presses:** The *grey vertical bar* represents the number of ethanol active lever presses after vehicle (aCSF) microinjection. *Blue vertical bars* represent the overall effect of NASPM (5.6 and 10.0 µg/0.5µL) on number of active lever presses for ethanol. **(C) Active cumulative responses:** *Grey circles* represent the cumulative number of sucrose active lever presses after vehicle (aCSF) microinjection. *Orange circles* represent the progressive effect of NASPM (5.6 and 10.0 µg/0.5µL) on the cumulative number of active lever presses for sucrose. **(D) Active lever presses:** The *grey vertical bar* represents the number of sucrose active lever presses after vehicle (aCSF) microinjection. *Orange vertical bars* represent the overall effect of NASPM (5.6 and 10.0 µg/0.5µL) on number of active lever presses for sucrose. In all panels, data are presented as mean (*vertical bars or circles*) ± SEM (*vertical lines*) and *asterisks* denote significance as compared to vehicle ($P<0.05$).

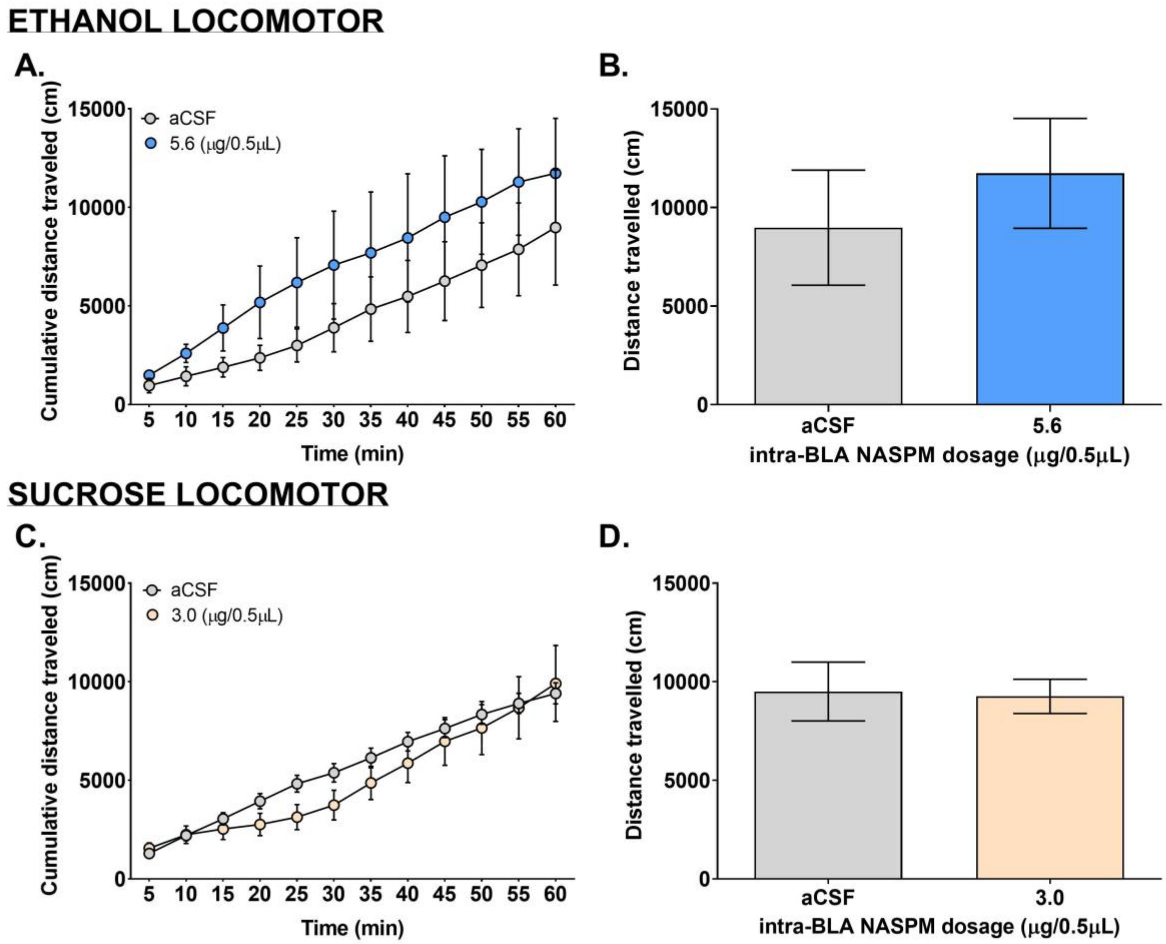

Figure 5. Locomotor activity after the microinjection of NASPM into the BLA
(A) Cumulative distance travelled: *Grey circles* represent the cumulative distance travelled (cm) after vehicle (aCSF) microinjection. *Blue circles* represent the progressive effect of NASPM (5.6 μg/0.5μL) on the cumulative distance travelled (cm). **(B) Total distance travelled:** The *grey vertical bar* represents the total distance travelled (cm) after vehicle (aCSF) microinjection. *Blue vertical bar* represents the overall effect of NASPM (5.6 μg/0.5μL) on total distance travelled (cm). **(C) Cumulative distance travelled:** *Grey circles* represent the cumulative distance travelled (cm) after vehicle (aCSF) microinjection. *Orange circles* represent the progressive effect of NASPM (3.0 μg/0.5μL) on the cumulative distance travelled (cm). **(D) Total distance travelled:** The *grey vertical bar* represents the total distance travelled (cm) after vehicle (aCSF) microinjection. *Orange vertical bar* represent the overall effect of NASPM (3.0 μg/0.5μL) on number of active lever presses for sucrose. In all panels, data are presented as mean (*vertical bars or circles*) ± SEM (*vertical lines*) and *asterisks* denote significance as compared to vehicle ($P<0.05$).

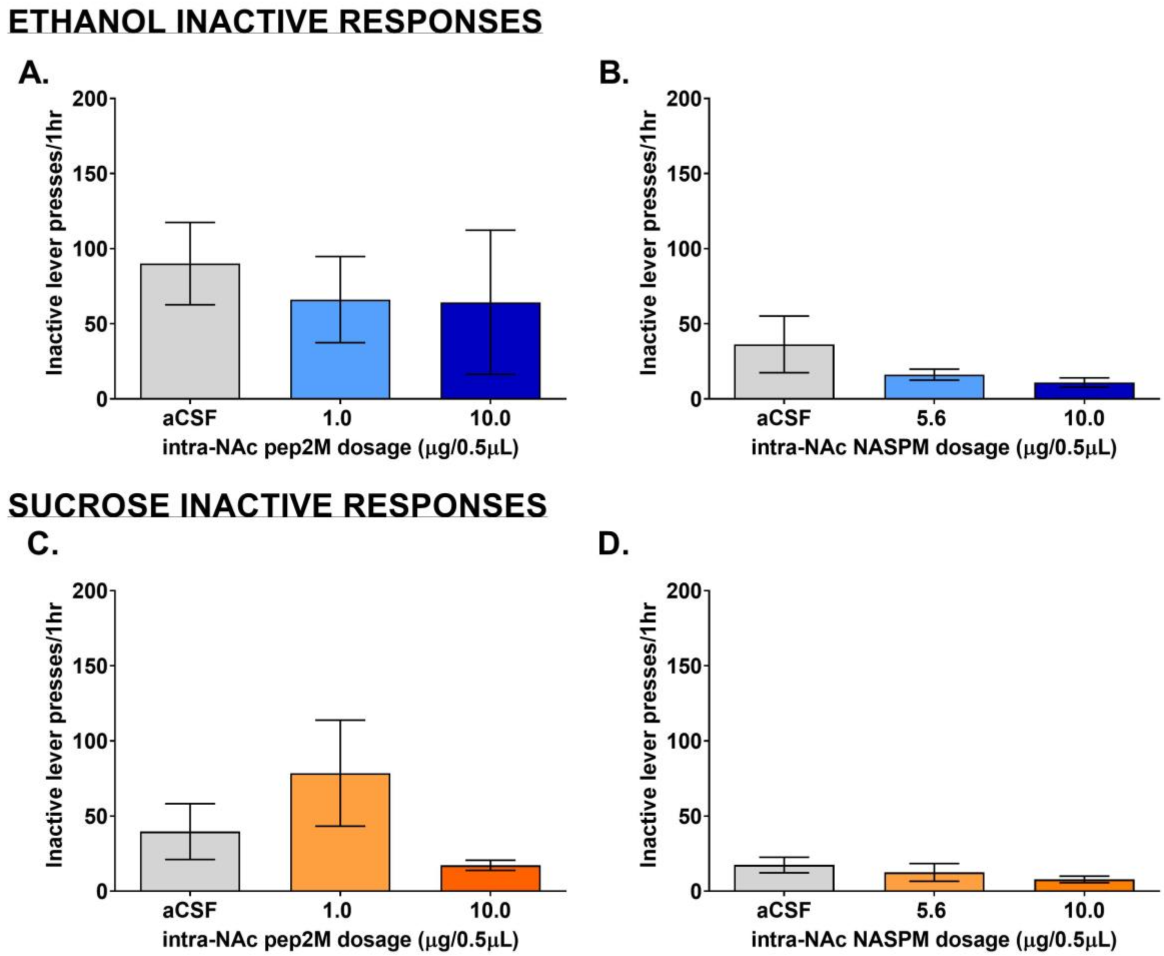

Figure 6. Inactive lever pressing after microinjection of pep2M in NAc or NASPM in BLA (A & C) pep2M in NAc: The *grey vertical bar* represents the number of ethanol or sucrose inactive lever presses after vehicle (aCSF) microinjection. *Blue vertical bars* represent the overall effect of pep2M (1.0 and 10.0 µg/0.5µL) on number of inactive lever presses for ethanol. *Orange vertical bars* represent the overall effect of pep2M (1.0 and 10.0 µg/0.5µL) on number of inactive lever presses for sucrose. **(B & D) NASPM in BLA:** The *grey vertical bar* represents the number of ethanol or sucrose inactive lever presses after vehicle (aCSF) microinjection. *Blue vertical bars* represent the overall effect of NASPM (5.6 and 10.0 µg/0.5µL) on number of inactive lever presses for ethanol. *Orange vertical bars* represent the overall effect of NASPM (5.6 and 10.0 µg/0.5µL) on number of inactive lever presses for sucrose. In all panels, data are presented as mean (*vertical bars or circles*) ± SEM (*vertical lines*) and *asterisks* denote significance as compared to vehicle ($P<0.05$).

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